*Strasbourg, April 9th 2014*

Dear Dr. Jaydev Upponi,

Thank you for your email and for the comments. We answer below and we have corrected the manuscript accordingly.

Could we schedule already the acquisition for the movie in Strasbourg ? Second half of june would be ideal for us.

With best regards,

Daniel Riveline

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CC: [elizabeth.sheeley@jove.com](mailto:elizabeth.sheeley@jove.com)

Dear Dr. Riveline,

Your manuscript JoVE51880R1 'Ordering cells and embryos in 3 dimensions: a new device for high content screening' has been peer-reviewed and the following comments need to be addressed.   
  
Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

**Editorial comments:**

*The editor made the following changes to the manuscript.*

*1.The editor moved “Check the power..” to the beginning of step 1.1.6.*

*2.The editor changed “Adapt the time..” to “Optimize the time..”.*

*3.The editor combined step 1.1.9 with 1.1.8 now, 1.1.8. Additionally, the editor removed the “Caution” sign below step 1.1.8 and combined “After the post-baking, cool the wafer..” with step 1.1.8.  
4.The editor renumbered step 1.1.9 and 1.1.10.*

*5.The editor un-highlighted the heading “Strategy 1”.*

*6.In step 1.3.1.7, the editor replaced “Note” with “Caution!”.*

*7.The editor removed the “Caution” sign and combined the statement with step 1.3.2.7.*

*8.The editor added “of fibronectin solution” in step 2.3*

*9.The editor combined the step 2.9 and 2.10, now step 2.9*

*10.The editor mentioned “see step 4.1” in step 2.16.*

*11.The editor combine step 3.1 and 3.2, now 3.1 and adjusted the numbering of the steps below.*

*12.In the paragraph below step 3, the editor removed all the commercial names such as Nikon and Leica. Similarly below step 5.*

*13.The editor highlighted step 3.1 for better continuity.*

*14.In step 4.1, the editor combined very small steps such that each step has 2-3 action items.*

*15.The editor combined step 5.1.2 and 5.1.3, now, 5.1.2. Similarly, 5.1.6 , 5.1.7 and 5.1.8, now 5.1.6. Similarly, from 5.2.4-5.2.8, now step 5.2.4.*

*16.Keeping the length of the highlighted section in mind and over all continuity, the editor un-highlighted the steps below protocol step 5*

**Answer:** We implemented all these changes and we agree with these modifications.

*Please retain all the above changes and please address the following comments.*

*1) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.*

**Answer:** The new version of the manuscript was read carefully and additionally the following changes were made:

1. The words ‘coined’, ‘glass’ of ‘glass coverslip’, ‘and’ of ‘Cavities were covered with fibronectin to facilitate adhesion and cells were inserted by centrifugation.’, ‘and’ of ‘Filling percentage was optimized for each system allowing up to 80% and cells and embryos viability was confirmed.’ were deleted and ‘for the study of active processes’ was replaced by ‘to study active processes’. All changes were done to reduce the length of the abstract to 300 words.
2. The word ‘release’ was replaced by ‘Remove’ in step 4.1.1.
3. The sentence in Page 10, Line 331: ‘Alternatively an upright microscope was used,’ was changed to ‘Alternatively an upright confocal microscope was used’.
4. In agreement with the editorial comment no 12, the commercial names in page 13, section 4.3 were removed and the paragraph was changed to: ‘For this example an upright confocal microscope is used, equipped with PMT and Hybrid detectors. A 25X or 63X HCX IR APO L water objective (0.95 NA,) was selected to provide a wide field of the sample and show the applicability of the device for high-content-screening applications’. Similarly, the commercial names in page 14, section 5.2, line 464 were removed and the sentence was changed to: ‘The observation was performed using an inverted phase-contrast microscope equipped with a 40X air objective 0.55 NA.
5. The sentence in Page 13, section 5.1, line 420 was changed to: ‘For fission yeast observation an inverted spinning disk confocal microscope was used.’

*2) Please keep in mind that we do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.*

**Answer:** In this work, we show representative results that demonstrate the strength of our method. Our claims are supported by data and by published references.

***Reviewers' comments:***

***Reviewer #1:***

*Title:*

*"Ordering cells and embryos in 3 dimensions: a new device for high content screening"  
The title is confusing. It can be expected that they present a method to ordering cells in 3 dimensions. From the manuscript, they show a method to ordering cells in a 2D array. The point is that each individual cell is located in an individual 3D well.*

*This should be clarified in the title and the others parts of the manuscript.*

**Answer:** We agree with the reviewer and we changed the title for: *''Ordering single cells and single embryos in 3D confinement: a new device for high content screening''*. The addition of the word 'single' highlights the fact that individual cells/embryos are located in an individual 3D well with orientation of the ring for example. We also took into account the comment of Reviewer #2 (see below).

*Short abstract:*

*The authors claim "Their 3D confinement mimics physiological conditions". In my opinion the 3D wells allows a 3D environment for the cells. This is an important point to study but the 3D wells do not "mimics physiological conditions" as stated by the authors. Among others, cell mechanotransduction or cell-cell communication can also play an important role.*

**Answer:** We agree that the statement can be formulated more specifically. We changed the sentence for: "*Their 3D confinement is a step towards 3D environments encountered in physiological conditions*". Page 2, Lines 47-49.

*Abstract:*

*The claim "This device consists of an array of microcavities" It would be better to change to "This device consists of a 2D array of microcavities".*

**Answer:** The sentence was changed accordingly. Page 2, Lines 61

*Protocol:*

*Page 4 "Place the wafer with the photoresist layer with circular features of 20 μm (see Note) in diameter into contact with the photolithographic mask on the mask aligner" This sentence is confusing.*

**Answer:** The sentence was modified for: ´Load the wafer on the mask aligner for UV exposure. Place the photolithography mask on it. The mask shows a pattern of circular features (disks) of 20 m in diameter´. Page 4, Lines 136-138 in step 1.1.5.

*Page 4 "…NOTE: Diameters of disks should.." define disks.*

**Answer:** We define disks: 'Dimensions of disks dimensions on the mask will determine the diameter of cavities in the device'. Page 5, Lines 144-145, below step 1.1.5.

*Page 13 "These conical 'eggcups' can be fabricated by means of Deep Reactive Ion Etching technology in a specialized microfabrication center." Explain better how to produce the conical eggcups*

**Answer:** This is a standard microfabrication procedure which, for the sake of simplicity, we prefer not to detail the protocol in length and prefer to refer to the published patent and references therein, on Page 13, Line 433 (ref. 13) in the Note of step 5.1.1.

*Representative Results.*

*The authors claim "The 'eggcups' are a novel high-content-screening methodology which allows the visualization of oriented cells and embryos in a 3D physiological environment". It would better to change "3D physiological environment" by "3D environment". In the physiological environment the mechanical properties of the surrounding environment of the cells can also play a role.*

**Answer:** We changed the sentence along the suggestion of the referee, on Page 15, Line 483.

***Reviewer #2:***

*Manuscript Summary:*

*Review: JoVE51880R1 - Ordering cells and embryos in 3 dimensions: a new device for high content screening, Wollrab et. al.*

*The authors describe a method for the production of micro-well arrays for confinement of cells and embryos for screening applications. The manuscript is well presented and describes the method of production of the micro-wells and the subsequent usage for screening experiments in a way suitable for a researcher in the field to replicate the experiment, providing a useful technique for both single cell/embryo analysis and population studies. The work completed is accurate and complies with research standards for cell analysis. The manuscript would benefit from the addition of more background on some of the techniques used (e.g. photolithography and soft lithography) and an examination of competing technologies.*

**Answer:** We thank the reviewer for these comments. We added some elements along this line of suggestions (see below).

*Major Concerns:*

*Points of note:*

*\* I am not sure that the title accurately reflects the work completed. "Ordering of the cells and embryos in 3 dimensions: …" suggests a fully 3-D ordering of the cells where cells are arranged in the z- direction as well as the x- and y-. In this work, the cells are ordered in a 2-D array (x- and y- only), although individual cells are confined in a way that more closely resembles the 3-D physiology of the cell in the body, compared to 2-D surface culture. As such, I would suggest that the authors reconsider the title to more accurately reflect the work. My suggestion would be something along the lines of "Ordered micro-well arrays for 3-dimensional confinement of cells and embryos: a new device for high content screening".*

**Answer:** We agree with the reviewer, although the suggested title does not reflect the proper orientation of the cytokinetic ring for example. We propose: ´Ordering single cells and single embryos in 3D confinement: a new device for high content screening´.

*\* In the abstract, the authors refer to the "identification of features" using the micro-wells. The authors should expand on this to give an example(s) so that someone reading the abstract has an indication of these features.*

**Answer:** We changed the sentence for: "This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure and compacted phenotypes for Golgi and nucleus alignment". We have included this on Page 3, Lines 70-71 .

*\* In the short abstract, the authors describe the work as the production of a "new" device for cell culture. Others have previously reported on the production of micro-wells for cell culture (e.g. reference 11) therefore I would suggest the authors define the novelty of their devices, or remove the "new".*

**Answer:** We changed the sentence for: '*a device and a new method'*. The novelty in orientation is detailed in the next sentence of the short abstract on Page 2, Short abstract.

*\* A small discussion on the choice of the dimensions for the micro-wells would be helpful. Is there a way of defining well sizes that accommodate the cells? The authors state that the wells can be "adapted" (line 625), but how much adaptation is required, and how much can the micro-wells be designed for different cell types/embryos?*

**Answer:** We added the following sentences. ´The dimensions of ´eggcups´ were selected in the following manner: dimensions of cells which undergo division were measured on 2D surfaces: they have a spherical shape and their diameter was taken as a good indication for the EC diameter. Cells in ´eggcups´ elongate and orient along their long axis during cell division for example. This dimension depends on the system - cells and embryos - so this dimension should be evaluated in each case.' We have implemented this on Page 15, Lines 490-496.

*\* In a similar vein, if the well sizes are not a good fit for the dimensions of the cell, what effects does this have on the subsequent culture experiments? Does a "badly fitting" well start to approximate to a 2-D surface rather than a 3-D confinement? How can the culture experiment compensate for dimensional irregularity? If the cell does not "fit" into the micro-well, there will still be some scope for "stress". Is it that there has to be some compromise between stress and conformity?*

**Answer:** If well diameters are too small, cells/embryos do not enter. If they are too large, the orientation of organelles will not be observed. The challenge and novelty consist in matching the cell/embryo dimensions with the micro-well diameter.

*\* I cannot find Trimethylchlorosilane (TMCS) in the materials list. The authors should include a warning about the use of TMCS in the text. TMCS produces acute inhalation and dermal toxicity, and is highly flammable (with ignition flashback able to occur across considerable distances), consequently it should be used in a fume cupboard away from sources of ignition.*

**Answer:** These elements are added in the new version of the materials list. We have also included a warning about the use of TMCS in the main manuscript on Page 7, Lines 211-213..

\* It is my understanding that the production of the PDMS depends on the manufacturers recipe, and that different recipes give PDMS with different properties (elasticity, surface hydrophobicity etc.). The properties of the PDMS are not discussed in the manuscript, but may be relevant for the later functionalization and culture studies. Can the authors comment on the properties of the PDMS used here, and the variety of PDMS types available, and refer the reader to the manufacturer's specifications in each case.

**Answer:** The PDMS commonly used comes from Dow Corning. To our knowledge, the product is pretty robust, and most of the laboratories currently working in this field (*e.g.* G.W. Whitesides, M. Piel, M. Thery, B. Ladoux…) usually mention the same standard protocol.

*\* Section 1.3.1: How do the authors ensure any residual TMCS silane does not affect the subsequent steps of the functionalization and culture?*

**Answer:** We thank the reviewer for this observation. We performed extensive washing steps in order to ensure that the functionalization of the PDMS stamp with TMCS did not affect the subsequent fibronectin adhesion and cell culture on the EC. The obtained results supported this; cell behavior was not affected, and in particular, division kinetics was the same as compared to standard 2D cultures. Additionally, we did not observe any difference between *Strategy 1* (using TMCS) and *Strategy 2* (using SigmacoteTM, another siliconizing agent) (see section 1.3.2). Both strategies, using different products to replicate the ´eggcups´, lead to the very same results. Altogether, cells are not affected by the treatment with TMCS. We have though, added a comment about cleaning the PDMS stamp on section 1.3.1.7 (Page 7, Line 233) and 1.3.2.10 (Page 8, Line 270).

*Minor Concerns:*

*Minor points:*

*\* The use of the term "egg-cups" is somewhat redundant as the field already has a term - micro-wells - which adequately describes the structures produced.*

**Answer:** We understand, but the word ´eggcups´ capture the novelty: matching the short axis of an ellipse to orient vertically individual cells/embryos. We would like to keep it.

*\* Can the authors refer to some literature work to give readers some background on the use and versatility of photolithography and soft lithography/hot embossing, especially in their use with respect biomedical applications? This will allow the reader access to more in-depth examinations of the respective fabrication techniques, underpinning the experiments described here.*

**Answer:** We now quote two new references (Ref. 15 and 16) in the Introduction section, on Page 43, Line 108:

1. Mehling and Tay, 2014, Curr Op Biotech **25**: 95-102.
2. Wolfe *et al*. 2010, Rapid Prototyping of Microstructures by Soft Lithography for Biotechnology, Microengineering in Biotechnology, Methods in Molecular Biology Volume 583, 2010, pp 81-107

*\* Can the authors give examples of some similar cell confinement systems already on the market, e.g. for small area/population cell culture or analysis*

**Answer:** We now quote micro-wells from other Companies on Page 20, Lines 702-703.

*\* The immobilisation of individual cells in a large array is extremely useful for the examination of populations of cells and their response to external stimuli. Can the authors give examples of literature population experiments that may utilise the micro-well arrays.*

**Answer:** We now quote the following papers at the end of the Introduction section, on Page 4, Lines119-121:

'Finally, our device will be useful for studying the distributions of cells responses to external stimuli, for example in cancer (Yao, X. et al. Integ Biol **6**, 388-398 (2014)) or in basic research (Eberwine et al., Nat Meth **11**, 25-27 (2014)).*'*

*\* Section 1.2.6: Does the PDMS need to be peeled off the substrate before cutting out, or should it be cut out and then peeled off?*

**Answer:** It should be cut first. This has been clarified on the note of section 1.2.6 on Page 6, Line 196.  
  
*\* Section 1.3.1.2: Can the authors define "upside"; is it the side with the structures on?*

**Answer:** We define it on Page 6, Line 205 as ´the side with the structures´.  
  
*\* Lines 712 and 722: Should "training" be replaced with "practice"?*

**Answer:** We agree with the referee. We have modified it accordingly, on Page 19, Line 656 and 657..  
  
*\* Line 151: should "appropriated" be replaced with "appropriate"?*

**Answer:** We agree with the referee. We have modified it accordingly, on Page 5, Line 156.  
  
  
  
*Additional Comments to Authors:*  
N/A  
  
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***Reviewer #3:***

*Manuscript Summary:*

*The paper reports a method to study cells and embryos in microcavities. The idea behind is to provide cells with a 3D microenvironment and by this mimic better the physiological culture conditions. The fabrication method is thoroughly explained and applied to a few examples: study of organelles and the closure of the cytokinetic ring during cell mitosis.*

**Answer:** We thank the reviewer for the comments and answer below.

*Major Concerns:*

*Although the paper deals with a very interesting topic my major concerns raise from its main focus. If it is a paper focused in the fabrication of the device then I am missing the added value of this device with other alternatives already found in literature such as the ones published by Kwon et at, Analytical Chemistry 2011 ir Khetani et al, Nature biotechnology 2008 to just cite some of them.*

**Answer:** The added values are connected to the 3D environment for single cells and embryos, and the orientation of cellular/embryos organelles compared to the quoted references.

*I think authors should comment more on the benefits of their approach (no need of clean room maybe? simplicity?) and comment more on their limitations.*

**Answer:** These points are now added in the MS. *'Our approach is simple and does not need a clean room*'. Page 4, Line 117 and Page 19, Lines 645-646.

*For instance, for how long can they have the cells in culture in such device?*

**Answer:** Cells can be cultured until confluence with this device. But the main interest is when individual cells/embryos are isolated within the cavities. This is specified on Page 20, Lines 675-677.

*In small cavities liquid handling and in particular medium exchange can be a problem and will for sure limit the kind of experiments that can be addressed using this platform.*

**Answer:** Since our device is 'open' above cavities, we checked that medium exchange was not a problem. Cells in EC did not show any degradation for both short and long-term experiments due to lack of medium exchange. In particular, cell cycle was not altered. This was added on Page 20, Lines 674-675..

*On the other hand, to apply this device in drug screening, how will be the controls defined?*

**Answer:** The controls will be defined as the mean signal/phenotypes of the read-out in the absence of drugs.

*Would that be in other (parallel) device?*

**Answer:** This could be in the same device in a multi-well plate for example. Since the MS is already long, we prefer to leave these issues for future developments of the method. However, we comment on the future developments of our assay on Page 20, Lines 697 and 698.

*As a lot of the process is performed manually, how the variability would affect control /sample in such cases?*

**Answer:** The device will be developed for industrial (drug screening) applications at the bottom of a multiwell plate. As such, high content screening assays will be performed following the current automatised processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability. This point is added on Page Page 20, Lines 697-699.

*Regarding the protocol itself I find some details missing. One important issue is sterility, how is that performed in such devices?*

*at which point of the protocol?*

**Answer:** Sterility of EC is ensured by UV exposure. This has been clarified on Page 9, Line 280, in section 2.3. Note also that a caution note about sterility was included on Page 9, Line 286 below section 2.5.

*The other issue is the time that cell should be into the microcavities before rinsing to remove unattached cells, I could not find it.*

**Answer:** Cells are 'captured' by the cavities either by their shapes (for fission yeast) or by adhesion molecules (for mammalian cells). The rinsing occurs immediately after centrifugation. This is described (and clarified) in section 2.13 on Page 10, Line 317-319.

*On the contrary, if the main focus of the paper is on the new applications of such device I find that the novelties should be properly remarked and discussed and this should be done starting with the abstract. In this they claim "This device allowed the identification of new features which were unknown by using standard 2D cell culture assays" but they do not give specific information to make such features relevant.*

**Answer:** We took the option to present the novelty of the approach with illustrations. As suggested by the referee, we have included the novelties in the abstract: ´This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure, compacted phenotypes for Golgi and nucleus alignment´. These changes have been implemented on Page 3, Lines 70-71.

*As the closure of the cytokinetic ring during cell mitosis has been already observed in similar devices by the same authors they should provide more information about what the new findings are.*

**Answer:** As agreed with the editor - this paper was an invitation by JoVE - this paper will appear after the publication of the main results. In this work (JoVE), we provide new findings about the different phenotypes of the Golgi apparatus and the study of the nucleus orientation. In addition, we give more information about the cytokinetic ring and the capabilities of the EC for being applied in different model systems (mammalian cells, yeast, C.*elegans*…) which has not been shown so far.

*Moreover, new applications of the device would probably need the mimicking of cell-cell and cell-ECM contacts. They just briefly menction this in the discussion and I would have loved to see how they envisage to include such modifications into the device.*

**Answer:** We have further developed this point: ´In this setup single cells are captured. This is in contrast to epithelial tissues encountered *in vivo*. However, this environment could be reproduced in our ‘eggcups’ by coating the side walls with cadherins to mimic cell-cell contacts using more flexible elastomers. Focal contacts will be promoted by the deposition of fibronectin at the bottom of the wells. These respective distributions of adhesion molecules should allow in reproducing the cellular environments encountered *in vivo*. By this method one would approach the physiological conditions.´

We have implemented this change on Page 19-20, Lines 668-674.

*Finally, high throughput techniques in the end should include some automatization in the fabrication processes but also in the visualization techniques and this may also be included or considered in this device.*

**Answer:** We agree with the referee.We now discuss on Page 20, Lines 698 and 699 the applicability of the ´eggcups´ for high-content-screening applications, in particular for industrial-oriented purposes:

´As such, high-content-screening assays will be performed using the commonly used automatised processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability.´

*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A  
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Your revision is due by **Apr 09, 2014**.  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove) and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.   
  
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